Biochimica et Biophysica Acta 1793 (2009) 1759–1767

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Visfatin through STAT3 activation enhances IL-6 expression that promotes endothelial angiogenesis

Jee-Young Kim [a,e,f,1], Yun-Hee Bae [a,e,f,1], Moon-Kyoung Bae [b], Su-Ryun Kim [c,d], Hyun-Joo Park [b], Hee-Jun Wee [d], Soo-Kyung Bae [a,e,f,*]

[a] Department of Physiology, School of Medicine, Yangsan Campus of Pusan National University, Yangsan, 626-870, South Korea
[b] Department of Oral Physiology, School of Dentistry, Yangsan Campus of Pusan National University, Yangsan, 626-870, South Korea
[c] Department of Molecular Biology, College of Natural Science, Pusan National University, Busan, 602-739, South Korea
[d] Department of Biochemistry, School of Medicine, Institute for Tumor Research, Chungbuk National University, Cheongju, 361-763, South Korea.
[e] Medical Research Center for Ischemic Tissue Regeneration, Pusan National University, Yangsan, 626-870, South Korea
[f] Pusan National University Medical Science Education Center (BK21 Program), Pusan National University, Yangsan, 626-870, South Korea

ARTICLE INFO

Article history:
Received 27 June 2009
Received in revised form 2 September 2009
Accepted 3 September 2009
Available online 12 September 2009

Keywords:
Visfatin
STAT3
IL-6
Angiogenesis
Human endothelial cell

ABSTRACT

Signal transducer and activator of transcription 3 (STAT3) acts as a mediator and biomarker in endothelial activation. We have recently shown that a novel adipokine visfatin promotes endothelial angiogenesis. The present study was to determine whether visfatin affects STAT3 activity and to explore the potential target gene(s). Here, we found that visfatin induced the activation of STAT3, as characterized by increased tyrosine phosphorylation, nuclear translocation, and DNA-binding activity in human endothelial cells. In addition, visfatin significantly upregulated mRNA and protein levels of endothelial interleukin-6 (IL-6), which was blocked by a specific inhibitor of STAT3 signaling and by the transfection of siRNA specific for STAT3. Furthermore, visfatin-induced angiogenesis was reduced by the inhibition of STAT3 signaling or neutralization of IL-6 function, as measured by tube formation, rat aortic ring assay, and mouse Matrigel plug assay. Taken together, our results provide the first example of STAT3-dependent endothelial IL-6 induction by visfatin and of the role of IL-6 in mediating visfatin-induced angiogenesis.

1. Introduction

Angiogenesis, the formation of new capillary blood vessels from existing microvessels, plays an important role in the process of embryonic development, and in adult tissue maintenance or regeneration [1,2]. Aberrant angiogenesis contributes to vascular diseases, including inflammation, atherosclerosis, tumor growth, and obesity [3]. Angiogenesis is modulated by a number of both positive and negative angiogenic factors with direct and indirect effects on endothelial cells [4]. Adipokines, biologically active polypeptide growth factors and cytokines that are mainly produced by adipose tissue and involved in obesity [5], have been reported to induce local and systemic angiogenesis [6].

Visfatin (also known as pre-B-cell colony-enhancing factor, PBEF), a highly conserved 52-kDa protein, is a novel adipokine that strongly correlates with visceral obesity [7,8]. We and others have previously demonstrated that visfatin has angiogenic activity in *in vitro*, *ex vivo*, and *in vivo* assays [9-11] and that visfatin upregulates the expression of FGF-2 and VEGF genes, leading to endothelial angiogenesis [11,12]. Thus, these results strongly indicate that visfatin acts as a pro-angiogenic adipokine via increasing levels of other angiogenic factors.

Interleukin-6 (IL-6), a multifunctional cytokine [13], induces angiogenesis *ex vivo* and *in vivo* [14]. In addition, *in vitro* studies have shown that IL-6 is capable to increase endothelial permeability and stimulates the proliferation of endothelial cells [15]. The levels of IL-6 are highly elevated in diseases that show dysregulated angiogenesis including arthritis, atherosclerosis, diabetes, cancer, and obesity [16,17], suggesting the importance of clarifying the mechanisms of IL-6 gene induction for therapeutic application against these vascular diseases.

So far, several nuclear transcription factors such as NF-IL6, AP1, and NF-kB have been reported to contribute to IL-6 gene induction [18]. For example, IL-1 and tumor necrosis factor alpha (TNF-α) induce IL-6 gene expression via NF-kB activation [19]. Recently, signal transducer and activator of transcription 3 (STAT3) was demonstrated to mediate IL-6 gene induction by phosphatidic acid and cardiotropin-1 in macrophage and human endothelial cells, respectively [20,21]. Moreover, studies have demonstrated the role of STAT3 as a mediator and biomarker in endothelial activation [22].

Here, we attempted to determine whether IL-6 gene expression is affected by visfatin in human endothelial cells, and to characterize the

* Corresponding author. Department of Physiology, School of Medicine, Pusan National University, Yangsan, 626-870, South Korea. Tel: +82 51 510 8074; fax: +82 51 510 8076.
E-mail address: skbae@pusan.ac.kr (S.-K. Bae).
[1] Denotes co-first authors.

0167-4889/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2009.09.006

mechanisms involved. We report for the first time that visfatin induces the activation of STAT3 upstream of IL-6 expression to induce angiogenesis (visfatin/STAT3/IL-6/angiogenesis).

2. Materials and methods

2.1. Reagents and recombinant proteins

Rabbit polyclonal anti-pSTAT3 and mouse monoclonal anti-α-Tubulin antibodies were obtained from Cell Signaling. Rabbit polyclonal anti-STAT3 antibody, HRP-conjugated goat anti-mouse IgG, and anti-rabbit IgG were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-IL-6 antibody was obtained from Abcam. FITC-conjugated donkey anti-rabbit IgG was purchased from Jackson Immunoresearch. Matrigel and α-Cyano-(3,4-dihydroxy)-N-benzylcinnamide (AG490) were from Calbiocam and BD Biotechnology, respectively. Recombinant human IL-6 was obtained from R and D systems. Recombinant human visfatin protein was prepared in our laboratory as previously described [9]. Polymyxin B (PMB) was obtained from Sigma.

2.2. Endothelial cell culture

Human dermal microvascular endothelial cells (HMECs) were obtained from the Center for Disease Control (CDC) in Atlanta, Georgia, USA. HMECs were maintained in MCDB 131 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% antibiotics, 10 mmol/l L-glutamine, 1 μg/ml hydrocortisone, and 10 ng/ml human EGF at 37 °C under a humidified 95% to 5% (vol/vol) mixture of air and CO_2. Primary human umbilical vein endothelial cells (HUVECs) (passages 5–8) were purchased from CLONECTICS. The HUVECs were plated onto 3% gelatin-coated dish and grown in M199 (Invitrogen) with heat-inactivated 20% FBS, 3 ng/ml bFGF, and 100 μg/ml heparin.

2.3. Transient transfection of siRNA

We designed and synthesized a double stranded siRNA oligonucleotide against STAT3 (5′-CAUCUGCCUAGAUCGGCUAdTdT-3′,3′-dTdTGUAGACGGAUCUAGCCGAU-5′) and negative-control siRNA was purchased from Bioneer. Oligofectamine (Invitrogen) was used

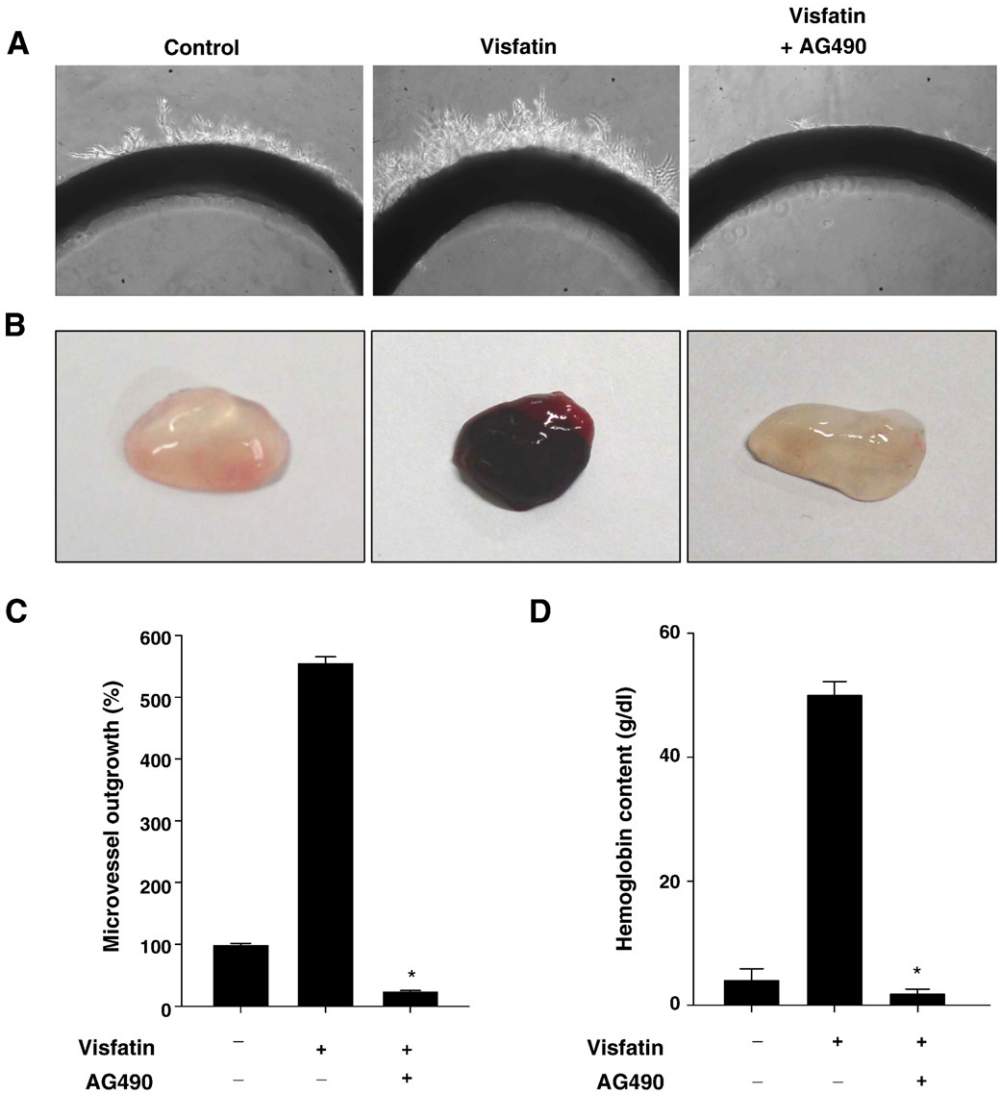

Fig. 1. JAK2-STAT3 signaling participates in visfatin-induced angiogenesis. (A, C) Rat aortic rings were embedded in Matrigel and cultured for 4 days with visfatin (500 ng/ml) in the presence or absence of AG490 (50 μM). Pictures are representative photographs for endothelial cell sprouts formed from the aortic ring segments (A). The degree of microvessel formation was quantified by counting the newly formed microvessels extending from the cultured aortic rings (C). (B, D) Matrigel plugs were photographed. Matrigel plugs mixed with visfatin (5 μg/ml) and AG490 (50 μM) were injected into mice (B). The concentration of hemoglobin was calculated from a parallel assay with a known amount of hemoglobin (D). *$P<0.01$ compared to visfatin.

as the transfection reagent as recommended by the manufacturer: 200 nM siRNA per 60 mm dish and 8 μl oligofectamine. HMECs were transfected at 30% confluence for 4 h with siRNA in minimal serum-free medium without antibiotics. Afterward, growth medium containing 3× the normal concentration of serum was added without removing the transfection mixture, and cells were allowed to grow an additional 44 h until confluent.

2.4. RNA extraction and RT-PCR analysis

Total RNA was harvest using TRIzol reagent (Invitrogen). cDNA was synthesized using MMLV reverse transcriptase (Promega) with 2 μg of each DNA-free total RNA sample and oligo(dT) as primer, according to the manufacturer's instructions. The oligonucleotide primers for PCR were designed as follows: β-actin, 5′-GACTACCTCATGAAGATC-3′ and 5′-GATCCACATCTGCTGGAA-3′; hIL-6, 5′-AGATTCCAAAGATGTAGCCG-3′ and 5′-TCTTTGCTGCTTTCACACAT-3′. RT-PCR products of IL-6 and β-actin were 146 bp and 507 bp, respectively. The amplification profile for IL-6 was 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s for 29 cycles. After the RT-PCR, the amplified products were separated on a 1.8% (wt/vol) agarose gel and visualized by ethidium bromide staining under UV light illumination.

2.5. Western immunoblot analysis

Harvested cells were lysed in a lysis buffer (40 mM Tris-Cl (pH 7.2), 10 mM EDTA, 120 mM NaCl, and 0.1% NP-40 with protease inhibitor cocktail) and protein levels were measured by BCA assay. A constant protein concentration (30 μg/lane) was used. The protein extracts were boiled for 5 min, loaded, separated by SDS/PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). The membranes were probed for a specific antibody binding according to manufacturers' recommended protocols for the individual antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech).

2.6. Nuclear extraction and EMSA

To prepare nuclear protein extracts, HMECs were washed with cold PBS (pH 7.4) and then immediately harvested by scraping. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were lysed in cold buffer A (pH 7.5) containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM NaCl, and 0.25% NP-40 for 5 min at 4 °C and then centrifuged at 4000 rpm for 2 min to obtain a nuclear pellet. The supernatant was removed and the nuclei pellets were resuspended in cold buffer C (pH 7.5) containing 20 mM HEPES, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl2, 10 mM NaCl, and 0.25% NP-40; vigorously agitated from time to time; and then centrifuged 14000 rpm for 5 min. The supernatant containing the nuclear proteins was diluted with cold buffer D (pH 7.5) containing 20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 20% glycerol and used for the EMSA. The STAT3 consensus oligonucleotide, 5′-GATCCTTCTGGGAATTCCTAGATC-3 was end labeled with biotin using a Biotin 3′ End DNA Labeling Kit (Pierce). Binding reactions containing equal amounts of protein (1 μg) and biotin-labeled STAT3 oligonucleotide (probe) were performed for 20 min in binding buffer (10 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40, and 2.5% glycerol). Binding reactions were analyzed using 6% native PAGE. After blotting to a nylon membrane, labeled oligonucleotides were detected with the Light Shift Chemiluminescent EMSA Kit following the instructions of the manufacturer (Pierce). Competitors were

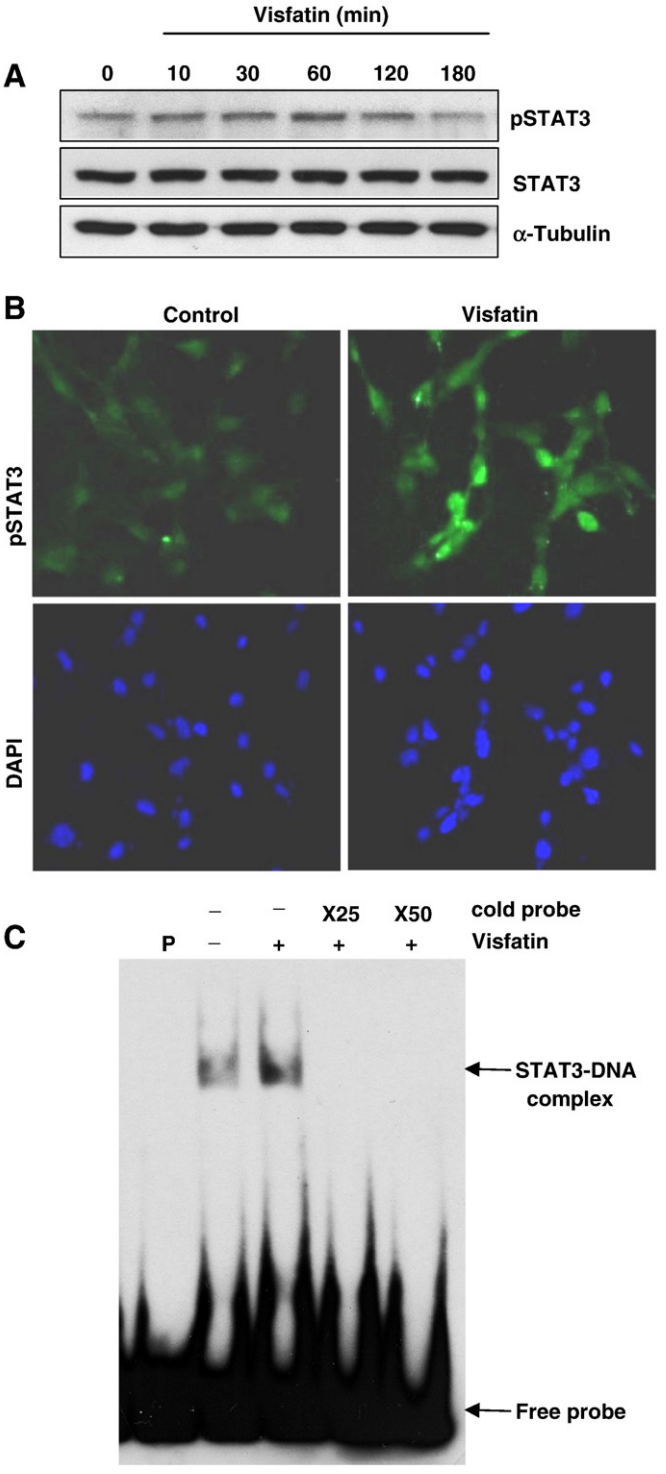

Fig. 2. Visfatin activates endothelial STAT3. HMECs were preincubated with the phosphatase inhibitor, NaF (5 mM) for 30 min prior to treatment with visfatin (500 ng/ml) and polymyxin B (10 μg/ml) for the indicated times. (A) Protein levels were examined by Western immunoblot assay using anti-STAT3, anti-phosphotyrosine (705)-STAT3, and anti-α-Tubulin antibodies. α-Tubulin served as the loading control. (B) Immunocytochemical analysis of pSTAT3 localization. HMECs were grown on cover slips and then stained with primary antibodies directed against pSTAT3 (Tyr705). Following treatment with DAPI (blue nuclear stain) and fluorescent secondary antibodies for pSTAT3 (green), the cells were examined using fluorescence microscopy. Overlapping of the green (pSTAT3) and the blue (nucleus) colors indicates the localization of activated STAT3 in the nuclei of HMECs. (C) The binding activity of STAT3 in nuclear extracts was measured by EMSA. Nuclear extracts from HMECs were incubated with biotin-labeled STAT3 oligonucleotide as a probe. Arrow indicates the migration of STAT3 protein—DNA complex (lanes 2, 3). Specificity is indicated by competition analysis with 'cold' STAT3 probe. In the competition assay, 25- and 50-fold molar excess unlabeled probe were added to the reaction mixtures (lanes 4, 5).

generated by annealing the unlabelled sense and antisense oligonucleotides, and were added at 25- and 50-fold molar excess to confirm the identities of the protein/DNA complexes.

2.7. ELISA

The amounts of IL-6 in media from HMECs stimulated with visfatin were determined by specific enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BioLegend). The absorbance (450 nm) for each sample was analyzed using an ELISA reader (EB800, Bio-Tek Instruments) and was interpolated with a standard curve.

2.8. Immunocytochemistry

HMECs cultured on a coverglass were fixed in 4% paraformaldehyde in PBS for 10 min and covered with ice-cold 100% methanol for 10 min, then rinsed in PBS for 5 min. The cells were blocked with 0.3% Triton X-100/5% normal goat serum/PBS for 60 min, and then labeled with the primary antibody (rabbit polyclonal anti-STAT3 antibody). After overnight incubation at 4 °C, the cells were incubated with FITC-conjugated secondary antibody for 90 min. Coverslips were mounted in Vectastain containing DAPI (Vector Laboratories). Cells were analyzed using fluorescence microscopy (Nikon).

2.9 In vitro tube formation assay

HMECs were seeded in medium containing 1% FBS in ultra low attachment plates (6-well plates) and incubated with neutralizing antibody against human IL-6 (200 μg/ml) for 1 h. 48-well culture plates were coated with growth factor-reduced Matrigel in a total volume of 130 μl per well and allowed to solidify for 30 min at 37 °C. Cells in 6-well plates were transferred into the 48-well plates and visfatin (500 ng/ml) was added to the each well. The plates incubated for 8 h at 37 °C and morphological changes of the cells were photographed under a microscope.

2.10. In vivo Matrigel plug assay

Angiogenesis was quantified by the matrigel plug assay as previously described [10]. Briefly, matrigel, in liquid from at 4 °C, was mixed with visfatin (5 μg/ml) with and without AG490 (50 μM), and resulting mixture was injected (0.5 ml) into the abdominal subcutaneous tissue of 7-week-old male C57BL/6 mice, along the peritoneal midline (4 mice/group). Mice were killed 7 days later, and the matrigel plugs were removed and photographed. For hemoglobin determination, the plugs were homogenized in 500 μl distilled water and hemoglobin concentrations were measured using Drabkin reagent kit 525 (Sigma) for the quantification of blood vessel formation. All animal studies were conducted in accordance with our institutional

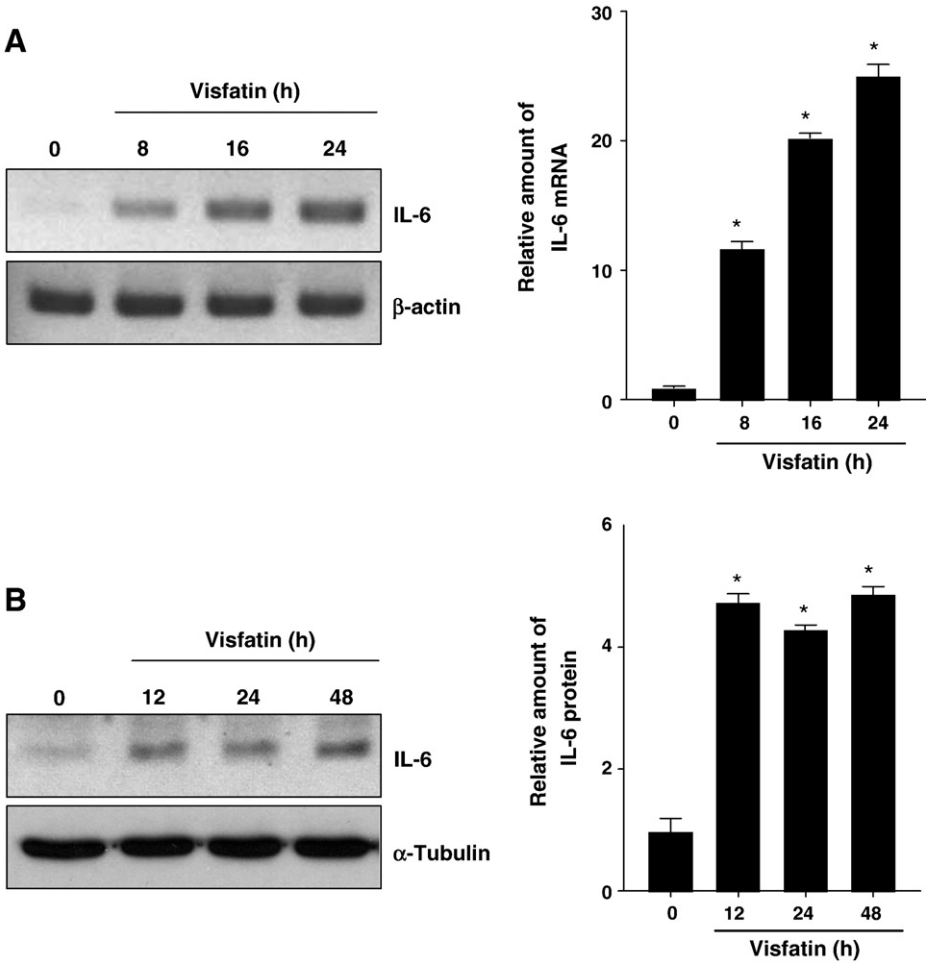

Fig. 3. Visfatin upregulates IL-6 production. HMECs were incubated with 500 ng/ml of visfatin for the indicated times. Culture medium was supplemented with polymyxin B (10 μg/ml). (A) Total RNAs were isolated and then analyzed by RT-PCR using specific primers for human IL-6 and β-actin. Quantitative analysis is shown on the lower panel. The indicated relative gene expression of IL-6 was normalized to the internal control, β-actin. (B) IL-6 protein levels were examined by Western immunoblot assay with antibodies against IL-6 and α-Tubulin antibodies. α-Tubulin served as the loading control. Quantitative analysis of pSTAT3 normalized to the STAT3 expression is shown on right panel. *$P<0.01$ compared to control.

guidelines for animal care and with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23 revised 1996), and approved by the Institutional Animal Care and Use Committee at Pusan National University, Korea.

2.11. Ex vivo rat aortic ring assay

Male Sprague–Dawley rats (SD, 6 weeks of age), weighing 210–230 g, were obtained from Samtako (Korea). The thoracic and abdominal aortas were dissected from rats and excess perivascular tissue was removed. Transverse sections (1–2 mm) were made, and the resulting aortic rings were then washed in medium 199 (Invitrogen). The aorta rings were placed on Matrigel-coated wells, covered with additional Matrigel, and allowed to gel for 30 min at 37 °C. Matrigel was allowed to congeal and was then covered with conditioned medium. Embedded aortic rings were cultured at 37 °C, 5% CO_2. On day 4, microvessel outgrowth was photographed under a phase contrast microscope.

3. Results

3.1. The JAK2-STAT3 signaling pathway is involved in visfatin-induced angiogenesis ex vivo and in vivo

In an initial screen of pharmacological inhibitors, AG490, a specific inhibitor of JAK2-STAT3 signaling [23], inhibited visfatin-induced angiogenesis in an *ex vivo* rat aorta model. Rat aortic rings were placed on Matrigel and incubated with visfatin in the absence or presence of AG490. Visfatin increased microvessel sprouting ~5.5-fold from the adventitia of aortic rings, but AG490 inhibited this outgrowth (Fig. 1A, C). Similar results were obtained by *in vivo* mouse Matrigel plug assay. Visfatin increased the hemoglobin content of Matrigel plugs in mice, a measure of angiogenesis, and AG490 blocked this increase (Fig. 1B, D). These results suggest that JAK2-STAT3 signaling may mediate visfatin-induced angiogenesis *ex vivo* and *in vivo*.

3.2. STAT3 is activated by visfatin in HMECs

To examine whether visfatin could activate STAT3 in endothelial cells, we measured STAT3 phosphorylation on tyrosine 705 in human microvessel endothelial cells (HMECs). Visfatin time-dependently stimulated tyrosine phosphorylation of STAT3 with a peak at 60 minutes (~2-fold) returning to basal levels by 180 min, but did not change levels of total STAT3 or control protein (α-Tubulin) (Fig. 2A). STAT3 phosphorylation determines the activation of STAT3, and upon activation, STAT3 dimerizes and subsequently translocates into the nucleus [24]. Thus, we examined the localization of pSTAT3 in visfatin-treated cells by immunocytochemistry. Visfatin treatment produced more STAT3-positive nuclei than controls (Fig. 2B). Active STAT3 proteins in the nucleus bind to specific sequences on target gene promoters to activate transcription [24]. Therefore, we checked the DNA binding activity of STAT3 in nuclear extracts of visfatin-treated cells by an electrophoretic mobility shift assay using biotin-labeled oligonucleotides as a specific probe for the STAT3 binding site. Visfatin stimulated the binding of nuclear extracts to the consensus STAT3 binding sequence (Fig. 2C, lane 2 and 3). This STAT3-probe complex was completely disrupted by competition with an unlabeled probe (Fig. 2C, lane 4 and 5), indicating binding specificity. Taken together, these results suggest that visfatin induces the activation of STAT3 in human endothelial cells.

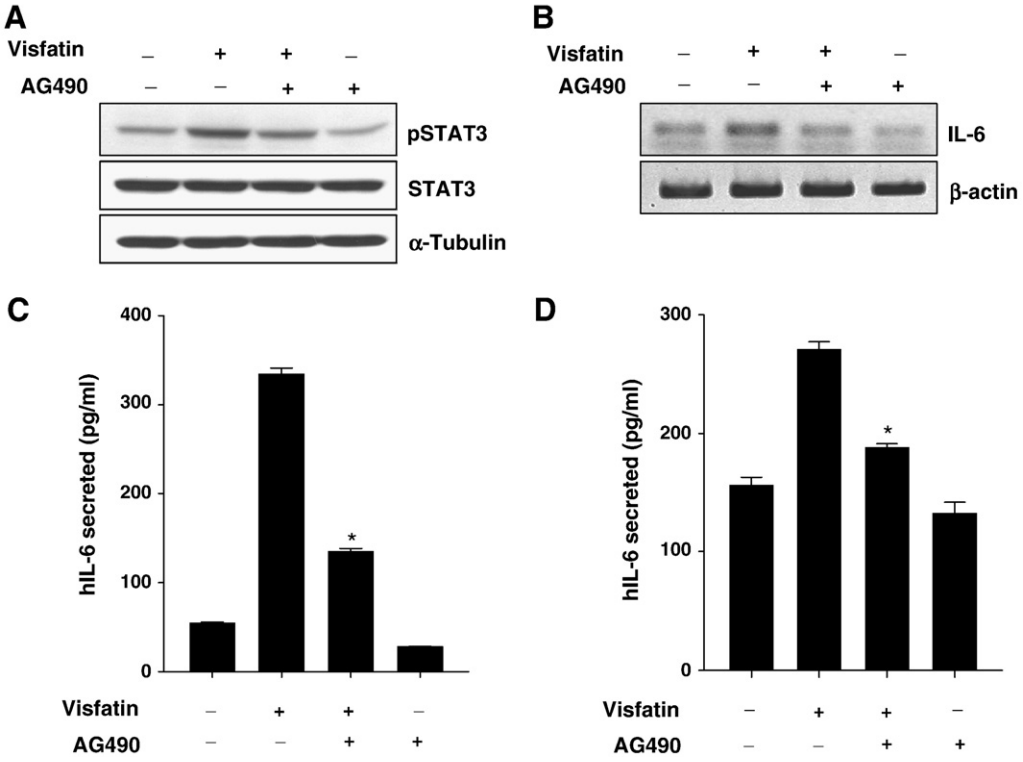

Fig. 4. STAT3 signaling pathway participates in visfatin-induced IL-6 production. (A) HMECs were preincubated with AG490 (25 μM) for 1 h before exposure to visfatin (500 ng/ml) and polymyxin B (10 μg/ml). The activation state of STAT3 was determined by Western immunoblot assay using anti-STAT3, anti-phosphotyrosine (705)-STAT3 and anti-α-Tubulin antibodies. α-Tubulin served as the loading control. (B) HMECs were preincubated with AG490 (25 μM) for 1 h before incubating with visfatin (500 ng/ml) and polymyxin B (10 μg/ml) for 24 h. The mRNA levels were analyzed by RT-PCR using specific primers for human IL-6 and β-actin. (C, D) HMECs (C) and HUVECs (D) were incubated with visfatin (500 ng/ml) for 24 h in the presence or absence of AG490 (25 μM). Culture medium was supplemented with polymyxin B (10 μg/ml). The amount of IL-6 protein secreted was measured by ELISA. *$P<0.01$ compared to visfatin.

3.3. IL-6 production is upregulated by visfatin in HMECs

IL-6, a multifunctional cytokine having angiogenic activity [13,14], has been recently reported to be one of STAT3 target genes [20,21]. Since, as shown in Fig. 2, STAT3 was activated by visfatin, we speculated that IL-6 could be induced by visfatin through the endothelial STAT3 activation. To test this possibility, HMECs were treated with visfatin, and IL-6 mRNA levels were analyzed by quantitative RT-PCR analysis. Visfatin upregulated the levels of IL-6 mRNA by ~25-fold in a time-dependent manner over the untreated control (Fig. 3A). The levels of IL-6 protein in visfatin-treated HMECs were further analyzed by Western immunoblot analysis and ELISA. Consistent with the RT-PCR findings, IL-6 protein levels were increased in cell lysates by ~4.9-fold after visfatin treatment (Fig. 3B). In addition, the release of IL-6 protein in the culture medium was enhanced by visfatin treatment (Fig. 4C), which corroborated the Western immunoblot analysis. Overall, these findings show that visfatin upregulates IL-6 gene expression in human endothelial cells.

3.4. STAT3 is involved in visfatin-induced IL-6 production in human endothelial cells

To elucidate whether expression of phosphorylated STAT3 is important for visfatin-induced IL-6 production, HMECs were pre-incubated with AG490 followed by visfatin treatment and then the levels of pSTAT3 protein, and IL-6 mRNA and protein were analyzed by Western immunoblot analysis and/or RT-PCR. Indeed, AG490 efficiently blocked visfatin-induced STAT3 phosphorylation (Fig. 4A) and impaired the induction of IL-6 gene expression by visfatin (Fig. 4B, C) in HMECs, suggesting that STAT3 activated by visfatin may contribute to IL-6 gene activation in human endothelial cells. Next, to investigate whether the stimulating effect of visfatin-induced STAT3 activation on IL-6 gene expression is dependent on endothelial cell types, human umbilical vein endothelial cells (HUVECs) were treated with visfatin in the presence or absence of AG490. Similarly, visfatin upregulated IL-6 protein level in HUVECs, which was blocked by AG490 (Fig. 4D), suggesting that mechanism(s) underlying visfatin-induced IL-6 gene activation via STAT3 may be very similar between microvascular EC and vein EC. Taken together, these results suggest that IL-6 upregulation by visfatin may be attributable to STAT3 activation in human endothelial cells.

3.5. STAT3 is required for visfatin-induced IL-6 production in human endothelial cells

To further verify the role of STAT3 in mediating visfatin-induced IL-6 gene activation, siRNA (short interference RNA) targeting for STAT3 was used to knockdown STAT3 expression in HMECs. The transfection of cells with STAT3 siRNA reduced both total STAT3 protein and visfatin-induced pSTAT3 levels (Fig. 5A), as well as the subsequent increases in IL-6 mRNA and protein (Fig. 5B and C). Overall, these findings show that STAT3 plays an important role in mediating visfatin-induced IL-6 production in HMECs.

3.6. IL-6 is involved in visfatin-induced angiogenesis in vitro and ex vivo

To determine whether IL-6 is required for visfatin-induced angiogenesis, we used anti-IL-6 neutralizing antibody to block IL-6 function in the *in vitro* tubular formation assay. Visfatin stimulated the formation of a capillary-like network of endothelial cells on Matrigel, but pretreatment with the neutralizing antibody blocked this activity (Fig. 6A and C). The anti-IL-6 neutralizing antibody also blocked visfatin-induced angiogenesis in an *ex vivo* aorta model of microvessel sprouting (Fig. 6B and D). The neutralizing antibody could block the activity of human recombinant IL-6 on aortic endothelial sprouting, validating its specificity. Taken together, these findings show that IL-6 mediates visfatin-induced angiogenesis *in vitro* and *ex vivo*.

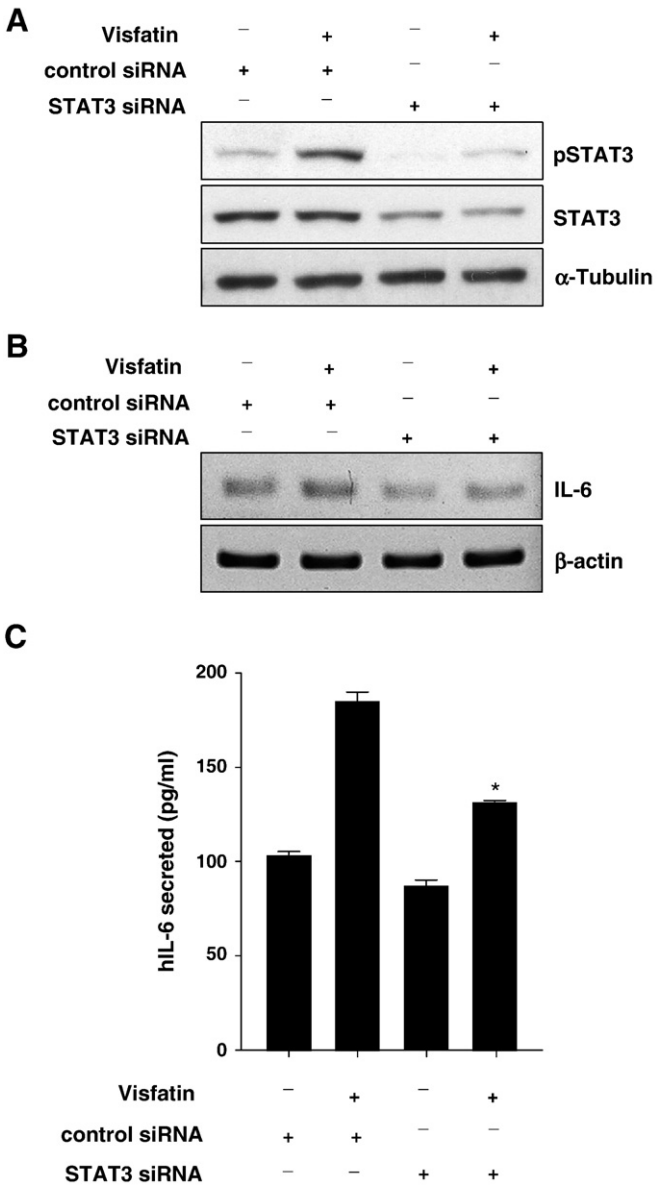

Fig. 5. STAT3 mediates visfatin-induced IL-6 production. HMECs were transfected with STAT3 siRNA (200 nM) or control siRNA (200 nM), according to the manufacturer's instructions. (A) Cells were incubated with visfatin (500 ng/ml) and polymyxin B (10 μg/ml) for 1 h before cell harvesting. The levels of STAT3 and pSTAT3 protein were determined by Western immunoblot assay using anti-STAT3, anti-phospotyrosine (705)-STAT3 and anti-α-Tubulin antibodies. α-Tubulin served as the loading control. (B, C) Cells were incubated with visfatin (500 ng/ml) and polymyxin B (10 μg/ml) for 24 h before cell harvesting. Total RNAs were isolated and then analyzed by RT-PCR using specific primers for human IL-6 and β-actin (B). β-actin served as an internal control. The amount of IL-6 protein secreted was measured by ELISA(C). *$P<0.01$ compared to visfatin-treated control siRNA.

4. Discussion

Recently, obesity has been considered to be a risk factor for tumor growth, vascular inflammation, cardiovascular diseases including atherosclerosis, and other angiogenic disorders [25,26] because many adipokines including leptin, resistin, VEGF, FGF-2, and IL-6 have activity to promote angiogenesis [6]. We and others have previously demonstrated that the adipokine visfatin stimulates endothelial angiogenesis and affects the expression of FGF-2 and VEGF genes in human endothelial

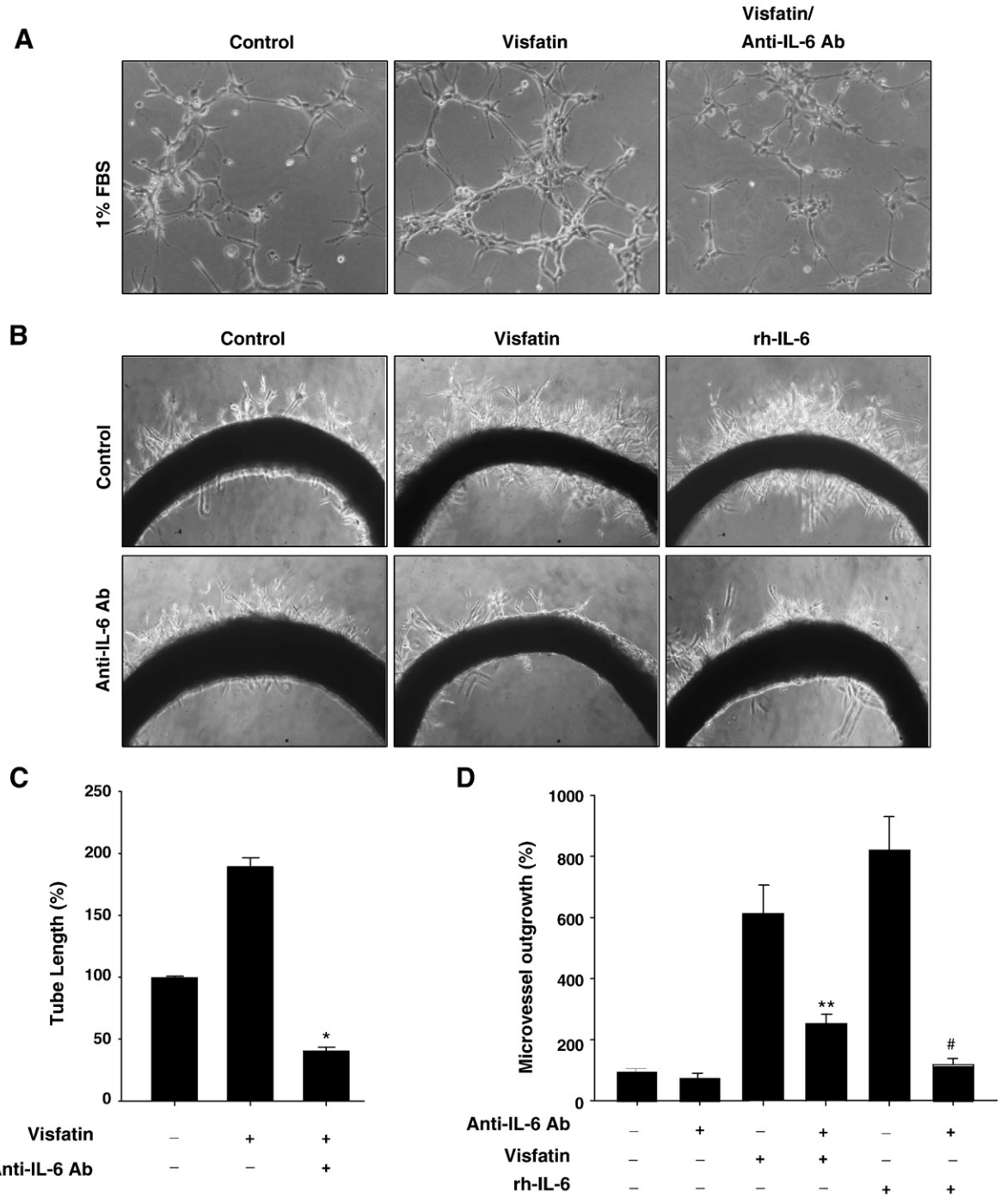

Fig. 6. IL-6 mediates visfatin-induced angiogenesis. (A, C) HMECs were incubated on the Matrigel with visfatin (500 ng/ml) after pretreatment with or without neutralizing antibody against IL-6 (200 μg/ml) for 1 h. Capillary-like tube formation was observed and photos were taken at 8 h after cells were plated onto Matrigel-coated plates (A). The quantitative tube formation assays are shown in the lower panel. The average numbers of tubule branch points were quantified (C). (B, D) Rat aortic rings were embedded into Matrigel and then treated with a combination of visfatin (500 ng/ml) and neutralizing antibody against IL-6 (400 μg/ml). Recombinant human IL-6 protein (50 ng/ml) was used as a positive control. On day 4, endothelial cell sprouts formed from the aortic ring segments were photographed using a phase contrast microscope (B). Quantitation of newly formed microvessels is shown in the lower panel. The total length of capillary-like tube was measured and normalized with controls (D). $^{**}P<0.05$, $^{*}P<0.01$ compared to visfatin, $^{\#}P<0.01$ compared to rh-IL-6.

cells [11,12]. Here, we have shown the following: (1) Visfatin induces the activation of STAT3 and upregulates endothelial IL-6 expression; (2) Blockade of STAT3 signaling inhibits visfatin-induced IL-6 expression; (3) Neutralization of IL-6 function suppresses visfatin-induced angiogenesis. Taken together, these findings show that STAT3 plays an important role in the induction of IL-6 by visfatin in human endothelial cells and reveal that IL-6 mediates visfatin-induced angiogenesis.

IL-6 is produced by various cells such as lymphocytes, synovial fibroblasts, macrophages, amniotic epithelial cells, tumor cells, and endothelial cells [16,17]. The released IL-6 form a complex with the IL-6 receptor (IL-6R), which is composed of IL-6Rα gp80 and the coreceptor gp130 and then activate JAK to phosphorylate tyrosine 705 on STAT3 [27]. The phosphorylated STAT3 (pSTAT3) dimerizes, subsequently translocates into the nucleus, and activates the transcription of its target genes [24,27], thereby regulating cell proliferation, differentiation, adhesion, migration, and apoptosis/survival [28]. However, in light of mounting evidence for the downstream signaling pathways of IL-6 and its role in various cells

including endothelial cells, little is known in regard to the mechanism of endothelial IL-6 gene induction. Here, we found that visfatin-activated STAT3 participates in the upregulation of IL-6 gene expression in human endothelial cells (Figs. 4 and 5). The role of STAT3 in the induction of IL-6 is further supported by other previous reports that phosphatidic acid can induce IL-6 synthesis through STAT3 activation in macrophages [20], and cardiotropin-1 activates STAT3 to increase IL-6 production in HUVECs [21]. The stimulating effect of visfatin on human IL-6 gene transcription via STAT3 was confirmed by human IL-6 gene promoter-driven reporter assay (data not shown). We used a reporter plasmid containing a 651-bp fragment of the human IL-6 gene promoter located directly upstream of the transcriptional start site and performed reporter assay after transfection of the plasmid into HMECs. In this preliminary study, we found that visfatin induced activation of a reporter plasmid containing a 651-bp fragment of the human IL-6 gene promoter located directly upstream of the transcriptional start site, and this could be blocked by AG490 (data not shown), suggesting that promoter activity of IL-6 gene is under the control of JAK2-STAT3 in HMECs, although it is unclear whether this control is mediated by direct binding of STAT3 on IL-6 promoter or indirectly.

We and others reported that PI3K, p38 MAPK, and ERK1/2 signaling pathways contribute to visfatin-induced angiogenesis [9,11,12,29] and showed that the NF-kB pathway is involved in visfatin activity [30-32]. We also observed that these pathways mediate IL-6 gene induction by visfatin in human endothelial cells (data not shown), consistent with a recent report [33]. Besides these pathways, in this study, our results add a new one, STAT3 signaling pathway, to the list of molecular mechanisms leading to visfatin-induced IL-6 upregulation (Figs. 4 and 5) and angiogenesis (Fig. 1).

STAT3 regulates cell proliferation and migration, and mediates vascular function. Furthermore, STAT3 is constitutively activated in many tumor cells and contributes to tumor cell proliferation and survival [22,34]. Therefore, considering the activity of visfatin to activate STAT3 (Fig. 2) and to induce angiogenesis (Figs. 1 and 5), it is likely that visfatin is able to promote the growth of cancer cells through the STAT3 activation, and can stimulate angiogenesis, potentially leading to tumorigenesis and tumor angiogenesis in STAT3-dependent human cancers, including gliomas and human breast tumors [35,36]. Supporting this, the role of visfatin in the development or progression of these cancers has been suggested by a recent report showing that visfatin serum levels were substantially elevated in many of astrocytoma and glioblastomas patients [37] and by our previous findings of strong visfatin immunostaining in human breast cancer tissues [38]. In addition, obesity has been reported to be a strong risk factor for many cancers including breast cancer [39]. Based on these findings, it appears that visfatin-STAT3 signaling functions as an important pathway operational in the pathogenesis of breast cancer disease in obese patients. This possibility is currently under investigation.

It is well known that IL-6 acts as a key proinflammatory cytokine to mediate both innate and acquired immune responses and has been linked to many kinds of inflammatory and cardiovascular diseases, including atherosclerosis, rheumatoid arthritis, diabetes, osteoporosis, and cancers [16,17,40]. Thus, the marked induction of IL-6 by visfatin suggests that visfatin could be an important modulator in these IL-6-related physiologic and/or pathophysiologic conditions. Indeed, recent evidence has shown that visfatin expression is highly detected in a variety of acute and chronic inflammatory diseases including sepsis, rheumatoid arthritis, inflammatory bowel disease, myocardial infarction, and atherosclerosis [41]. Considering that aberrant expression of IL-6 reportedly induces endothelial activation and leads to inflammation and vascular diseases [42], it is conceivable that visfatin could contribute to vascular inflammatory state, cardiovascular disease, and cancers, at least in part, by stimulating IL-6 gene expression. Therefore, our results in this study suggest that visfatin may aid in the development of these IL-6-associated physiologic and/or pathophysiologic conditions through positive feedback control of STAT3 activation, which is initially induced by visfatin and later by IL-6; namely, visfatin-STAT3-IL-6-STAT3. In this aspect, it will be interesting to study whether visfatin-STAT3-IL-6 signaling axis could be a new potential therapeutic target in these IL-6-associated diseases.

Recent studies have reported the role of visfatin-induced IL-6 in mediating anti-apoptosis [43]. For example, visfatin upregulates IL-6 protein level, which activates STAT3 signaling by binding of the IL-6 to IL-6 receptor, thereby protecting macrophages from ER stress-induced apoptosis [43]. Therefore, it is likely that visfatin-induced IL-6 may play a role in enhancing endothelial cell survival as well as in promoting angiogenic response.

Besides on IL-6, the stimulatory effects of visfatin on angiogenic factors have been recently reported by us and other investigators [10-12]. Production of human endothelial FGF-2 has been shown to increase after the treatment of visfatin [12]. In addition, VEGF levels were increased in visfatin-treated human endothelial cells [11]. Thus, visfain can be considered as a new proangiogenic adipokine, which is able to induce potent angiogenic factors, including FGF-2, VEGF and IL-6.

In summary, our findings provide the first example of STAT3-dependent endothelial IL-6 induction by visfatin and of the role of IL-6 in mediating visfatin-induced angiogenesis. These results provide novel insights into the mechanisms of vascular pathologies and new opportunities for therapeutic intervention by targeting visfatin/STAT3/IL-6 signaling axis.

Acknowledgements

This work was supported by the MRC program of MOST/KOSEF (R13-2005-009), the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-313-E00038) (to S.-K. Bae), and the research grant from a Vascular System Research Center grant from KOSEF (to M.-K. Bae).

References

[1] W. Risau, Mechanisms of angiogenesis, Nature 386 (1997) 671–674.
[2] P. Madeddu, Therapeutic angiogenesis and vasculogenesis for tissue regeneration, Exp. Physiol. 90 (2005) 315–326.
[3] N.M. Pandya, N.S. Dhalla, D.D. Santani, Angiogenesis—a new target for future therapy, Vascul. Pharmacol. 44 (2006) 265–274.
[4] Z.K. Otrock, R.A. Mahfouz, J.A. Makarem, A.I. Shamseddine, Understanding the biology of angiogenesis: review of the most important molecular mechanisms, Blood Cells Mol. Dis. 39 (2007) 212–220.
[5] R.S. Ahima, S.Y. Osei, Adipokines in obesity, Front. Horm. Res. 36 (2008) 182–197.
[6] Y. Cao, Angiogenesis modulates adipogenesis and obesity, J. Clin. Invest 117 (2007) 2362–2368.
[7] B. Samal, Y. Sun, G. Stearns, C. Xie, S. Suggs, I. McNiece, Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor, Mol. Cell. Biol. 14 (1994) 1431–1437.
[8] G. Sommer, A. Garten, S. Petzold, A.G. Beck-Sickinger, M. Bluher, M. Stumvoll, M. Fasshauer, Visfatin/PBEF/Nampt: structure, regulation and potential function of a novel adipokine, Clin. Sci. (Lond.) 115 (2008) 13–23.
[9] S.R. Kim, S.K. Bae, K.S. Choi, S.Y. Park, H.O. Jun, J.Y. Lee, H.O. Jang, I. Yun, K.H. Yoon, Y.J. Kim, M.A. Yoo, K.W. Kim, M.K. Bae, Visfatin promotes angiogenesis by activation of extracellular signal-regulated kinase 1/2, Biochem. Biophys. Res. Commun. 357 (2007) 150–156.
[10] R. Adya, B.K. Tan, J. Chen, H.S. Randeva, Pre-B cell colony enhancing factor (PBEF)/visfatin induces secretion of MCP-1 in human endothelial cells: Role in visfatin-induced angiogenesis, Atherosclerosis 205 (2009) 113–119.
[11] R. Adya, B.K. Tan, A. Punn, J. Chen, H.S. Randeva, Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis, Cardiovasc. Res. 78 (2008) 356–365.
[12] Y.H. Bae, M.K. Bae, S.R. Kim, J.H. Lee, H.J. Wee, S.K. Bae, Upregulation of fibroblast growth factor-2 by visfatin that promotes endothelial angiogenesis, Biochem. Biophys. Res. Commun. 379 (2009) 206–211.
[13] S. Akira, T. Hirano, T. Taga, T. Kishimoto, Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF), FASEB J. 4 (1990) 2860–2867.
[14] J. Hernandez-Rodriguez, M. Segarra, C. Vilardell, M. Sanchez, A. Garcia-Martinez, M.J. Esteban, J.M. Grau, A. Urbano-Márquez, D. Colomer, H.K. Kleinman, M.C. Cid, Elevated production of interleukin-6 is associated with a lower incidence of

disease-related ischemic events in patients with giant-cell arteritis: angiogenic activity of interleukin-6 as a potential protective mechanism, Circulation 107 (2003) 2428–2434.
[15] N. Maruo, I. Morita, M. Shirao, S. Murota, IL-6 increases endothelial permeability *in vitro*, Endocrinology 131 (1992) 710–714.
[16] D.A. Papanicolaou, R.L. Wilder, S.C. Manolagas, G.P. Chrousos, The pathophysiologic roles of interleukin-6 in human disease, Ann. Intern. Med. 128 (1998) 127–137.
[17] J.S. Yudkin, M. Kumari, S.E. Humphries, V. Mohamed-Ali, Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis 148 (2000) 209–214.
[18] J.M. Samuel, D. Kelberman, A.J. Smith, S.E. Humphries, P. Woo, Identification of a novel regulatory region in the interleukin-6 gene promoter, Cytokine 42 (2008) 256–264.
[19] Y.H. Zhang, J.X. Lin, J. Vilcek, Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence, Mol. Cell. Biol. 10 (1990) 3818–3823.
[20] C. Lee, H.K. Lim, J. Sakong, Y.S. Lee, J.R. Kim, S.H. Baek, Janus kinase-signal transducer and activator of transcription mediates phosphatidic acid-induced interleukin (IL)-1beta and IL-6 production, Mol. Pharmacol. 69 (2006) 1041–1047.
[21] M. Fritzenwanger, K. Meusel, M. Foerster, F. Kuethe, A. Krack, H.R. Figulla, Cardiotrophin-1 induces interleukin-6 synthesis in human umbilical vein endothelial cells, Cytokine 36 (2006) 101–106.
[22] Z. Chen, Z.C. Han, STAT3: a critical transcription activator in angiogenesis, Med. Res. Rev. 28 (2008) 185–200.
[23] N. Meydan, T. Grunberger, H. Dadi, M. Shahar, E. Arpaia, Z. Lapidot, J.S. Leeder, M. Freedman, A. Cohen, A. Gazit, A. Levitzki, C.M. Roifman, Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor, Nature 379 (1996) 645–648.
[24] J.N. Ihle, STATs: signal transducers and activators of transcription, Cell 84 (1996) 331–334.
[25] A.G. Renehan, D.L. Roberts, C. Dive, Obesity and cancer: pathophysiological and biological mechanisms, Arch. Physiol. Biochem. 114 (2008) 71–83.
[26] N. Ouchi, S. Kihara, S.T. Funahashi, Y. Matsuzawa, K. Walsh, Obesity, adiponectin and vascular inflammatory disease, Curr. Opin. Lipidol. 14 (2003) 561–566.
[27] P.C. Heinrich, I. Behrmann, G. Muller-Newen, F. Schaper, L. Graeve, Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway, Biochem. J. 334 (1998) 297–314.
[28] T. Hirano, K. Ishihara, M. Hibi, Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors, Oncogene 19 (2000) 2548–2556.
[29] F. Lovren, Y. Pan, P.C. Shukla, A. Quan, H. Teoh, P.E. Szmitko, M.D. Peterson, M. Gupta, M. Al-Omran, S. Verma, Visfatin activates eNOS via Akt and MAP kinases and improves endothelial function and angiogenesis *in vitro* and *in vivo*: translational implications for atherosclerosis, Am. J. Physiol. Endocrinol. Metab. 296 (2009) E1440–E1449.
[30] S.R. Kim, Y.H. Bae, S.K. Bae, K.S. Choi, K.H. Yoon, T.H. Koo, H.O. Jang, I. Yun, K.W. Kim, Y.G. Kwon, M.A. Yoo, M.K. Bae, Visfatin enhances ICAM-1 and VCAM-1 expression through ROS-dependent NF-kappaB activation in endothelial cells, Biochim. Biophys. Acta 1783 (2008) 886–895.
[31] W.J. Lee, C.S. Wu, H. Lin, I.T. Lee, C.M. Wu, J.J. Tseng, M.M. Chou, W.H. Sheu, Visfatin-induced expression of inflammatory mediators in human endothelial cells through the NF-kappaB pathway, Int. J. Obes. (Lond.) 33 (2009) 465–472.
[32] R. Adya, B.K. Tan, J. Chen, H.S. Randeva, Nuclear factor-kappaB induction by visfatin in human vascular endothelial cells: its role in MMP-2/9 production and activation, Diabetes Care 31 (2008) 758–760.
[33] S.W. Liu, S.B. Qiao, J.S. Yuan, D.Q. Liu, Visfatin stimulates production of monocyte chemotactic protein-1 and interleukin-6 in human vein umbilical endothelial cells, Horm. Metab. Res. 41 (2009) 281–286.
[34] J.F. Bromberg, M.H. Wrzeszczynska, G. Devgan, Y. Zhao, R.G. Pestell, C. Albanese, J.E. Darnell, Stat3 as an oncogene, Cell 98 (1999) 295–303.
[35] E.C. Brantley, E.N. Benveniste, Signal transducer and activator of transcription-3: a molecular hub for signaling pathways in gliomas, Mol. Cancer Res. 6 (2008) 675–684.
[36] S. Kunigal, S.S. Lakka, P.K. Sodadasu, N. Estes, J.S. Rao, Stat3-siRNA induces Fas-mediated apoptosis *in vitro* and *in vivo* in breast cancer, Int. J. Oncol. 34 (2009) 1209–1220.
[37] P.S. Reddy, S. Umesh, B. Thota, A. Tandon, P. Pandey, A.S. Hegde, A. Balasubramaniam, B.A. Chandramouli, V. Santosh, M.R. Rao, P. Kondaiah, K. Somasundaram, PBEF1/NAmPRTase/Visfatin: a potential malignant astrocytoma/glioblastoma serum marker with prognostic value, Cancer Biol. Ther. 7 (2008) 663–668.
[38] S.K. Bae, S.R. Kim, J.G. Kim, J.Y. Kim, T.H. Koo, H.O. Jang, I. Yun, M.A. Yoo, M.K. Bae, Hypoxic induction of human visfatin gene is directly mediated by hypoxia-inducible factor-1, FEBS Lett. 580 (2006) 4105–4113.
[39] G.D. Stephenson, D.P. Rose, Breast cancer and obesity: an update, Nutr. Cancer 45 (2003) 1–16.
[40] G.J. Zhang, I. Adachi, Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma, Anticancer Res. 19 (1999) 1427–1432.
[41] T. Luk, Z. Malam, J.C. Marshall, Pre-B cell colony-enhancing factor (PBEF)/visfatin: a novel mediator of innate immunity, J. Leukoc. Biol. 83 (2008) 804–816.
[42] V. Marin, F.A. Montero-Julian, S. Gres, V. Boulay, P. Bongrand, C. Farnarier, G. Kaplanski, The IL-6-soluble IL-6Ralpha autocrine loop of endothelial activation as an intermediate between acute and chronic inflammation: an experimental model involving thrombin, J. Immunol. 167 (2001) 3435–3442.
[43] Y. Li, Y. Zhang, B. Dorweiler, D. Cui, T. Wang, C.W. Woo, C.S. Brunkan, C. Wolberger, S. Imai, I. Tabas, Extracellular Nampt promotes macrophage survival via a nonenzymatic interleukin-6/STAT3 signaling mechanism, J. Biol. Chem. 283 (2008) 34833–34843.